

## Barley and Oat $\beta$ -Glucan Content Measured by Calcofluor Fluorescence in a Microplate Assay

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Of the various components of barley and oats, one constituent of the cereal cell wall, (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- $\beta$ -D-glucan ( $\beta$ -glucan), is important to a wide range of interests. Malting barley breeders strive to develop lines that modify well, yielding malts with low soluble  $\beta$ -glucan levels in Congress worts (less than the American Malting Barley Association guidelines of 100–120 ppm) (<http://www.ambainc.org/ni/Guidelines.pdf>) to meet brewers' needs for low-viscosity/high-filterability wort in the brewhouse. Similarly, barley grain with reduced  $\beta$ -glucan content is more suitable as poultry feed than barley because of a higher  $\beta$ -glucan content (Bergh et al 1999) due to feed digestibility and related issues. In contrast, the U.S. Food and Drug Administration recognizes benefits to human coronary health from including soluble  $\beta$ -glucan in the diet.  $\beta$ -glucan from oat and barley reduced both blood cholesterol levels as well as the glycemic index of foods (Wood 2002, Baik and Ullrich 2008).

With the increased interest in barley  $\beta$ -glucan as a food component, there is a need for convenient, cost-effective procedures to analyze the grain for  $\beta$ -glucan content. Probably the most widely-used method for analyzing barley (and oat)  $\beta$ -glucan content in research laboratories is the enzymatic method (McCleary and Codd, 1991) used in Approved Method 32-23 (AACC International 2000). Essentially, the mixed-linkage, linear  $\beta$ -glucan polymers are extracted from grain flour, hydrolyzed to monosaccharides by sequential enzymatic reactions (endo-(1 $\rightarrow$ 3,1 $\rightarrow$ 4)- $\beta$ -D-glucan 4-glucanohydrolase (lichenase), followed by  $\beta$ -glucosidase), and finally coupled to color generation using glucose oxidase and a chromogenic substrate. Kits providing the principle reagents for this method are commercially available from Megazyme ([www.megazyme.com](http://www.megazyme.com)) and require only relatively simple laboratory instruments (spectrophotometer, centrifuge, pipettes, water bath, etc.). However, the enzymatic method does include a number of manipulations, increasing the labor requirement for the method. Also, the costs for the reagent kits can be significant for large sample sets, making use of the kits less feasible for routine high-throughput analysis.

A second method for  $\beta$ -glucan determination measures fluorescence increase upon the binding of Calcofluor to high molecular weight  $\beta$ -glucans in grain extracts (Li et al 2008). Commonly, Calcofluor methods for  $\beta$ -glucan analysis involve use of flow injection analysis (FIA). In FIA, an autosampler is used to inject a fixed volume of sample into a stream of buffer or water, which is subsequently mixed with a second stream of Calcofluor reagent. A fluorescence detector measures the increase in fluorescence in

the sample bolus in the combined streams due to interaction of the sample  $\beta$ -glucan with the Calcofluor reagent. The sample  $\beta$ -glucan is quantified by comparing sample peak area or peak height with a standard curve generated from injections of a dilution series of  $\beta$ -glucan standards. This protocol, while procedurally simpler than the enzymatic method, requires more extensive instrumentation in its most common implementation.

Dedicated commercial instrumentation for such flow injection analysis or comparable segmented flow analysis can cost in excess of \$100,000. Home-built versions of such FIA instrumentation can be more affordable, although still requiring significant initial expense and maintenance costs. As a result, the FIA instrumentation is commonly found only in high-volume dedicated quality analysis laboratories. Apart from the cost of instrumentation, the Calcofluor methodology requires relatively inexpensive reagents and significantly less labor.

A third alternative method, using a dye-binding (colorimetric) procedure analogous to the Calcofluor method that can be read in a microplate spectrophotometer, has been adopted as EBC Method 4.16.3 High Molecular Weight  $\beta$ -Glucan Content of Wort: Colorimetric Method (see Frejje 2005). However, the kit for the colorimetric method (GlucaTest from NovaBiotec Dr. Fechter GmbH, [www.novabiotec.de](http://www.novabiotec.de)) is not commercially available in North America at the present time.

In this note, we show that the Calcofluor method can be adapted to a fluorescent microplate reader, providing a simple means of measuring  $\beta$ -glucan in cereal grains and malt, using inexpensive reagents, and affordable, commonly available instrumentation.

### MATERIALS AND METHODS.

Barley was malted as described by Schmitt and Budde (2007) and oats were malted at 16°C as described by Skoglund et al (2008). Grain and malt from barley and oat cultivars were extracted as described previously (Aastrup and Jørgensen 1988). After extraction, samples for FIA analysis were clarified by centrifugation and diluted (0.5 mL of sample + 2.5 mL of 200 mM pH 10.0 glycine buffer). Aliquots of the clarified grain and malt extracts for microplate analysis were analyzed without further dilution.

Analysis of extracted  $\beta$ -glucan generally followed the protocol for wort  $\beta$ -glucan determination presented previously (Schmitt and Budde 2009). Briefly, a series of aliquots (2–10  $\mu$ L for oat and barley grain, 20–60  $\mu$ L for malted barley and malted oat) of each extract was added to reagent-grade water in the wells of solid black 96-well microplates. Volumes of water added to individual wells were adjusted so that the volumes of sample + water totaled 100  $\mu$ L. Separately, a series of wells containing 0–1.75  $\mu$ g of barley  $\beta$ -D-glucan (Sigma) solution in 100  $\mu$ L of water plus  $\beta$ -glucan was prepared. When all water, sample, and standard additions were complete, 100  $\mu$ L of a working solution of 35 ppm Calcofluor in 0.01% Triton X-100 was rapidly added to each well on the plate using an 8-place electronic pipettor in multidispense mode. The plate was mixed well, and immediately read on a fluo-

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rescent plate reader (Gemini XS, Ex 365 nm, Em 420 nm, cutoff 420 nm, Molecular Devices). Relative fluorescent values for each well (samples and standards) were exported to Excel for subsequent data processing. Note that when pipetting such small volumes of samples and standards, careful pipetting technique and the use of high-quality pipettors and pipette tips is critical to accurate, repeatable analysis. In readers where the optical path passes through a liquid-air interface, as in the top-reading Gemini XS used in this study, inclusion of a surfactant such as the 0.01% Triton X-100 in the Calcofluor reagent is critical to assure uniform air-solution interfaces and, hence, consistent optical paths across sample and standard wells.

Where desired, samples can be checked for potential interferences by inclusion of internal standard  $\beta$ -glucan additions, allowing identification and correction of sample quenching that may be present (Schmitt and Budde 2009). Such internal standard calibrations can be constructed by generating orthogonal gradients of sample and internal standard additions across the 96-well plate such that the wells contain distinct combinations of  $\beta$ -glucan from sample and standard additions. Details for constructing the crossed-gradient plate layouts are available in the online e-Xtras for Schmitt and Budde (2009).

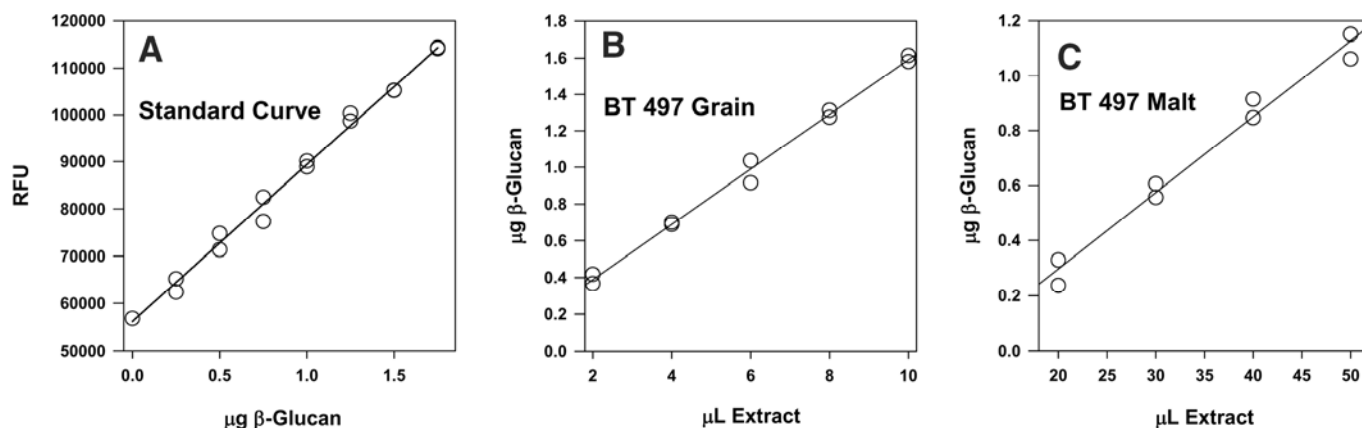
## RESULTS AND DISCUSSION

In previous studies for wort (Schmitt and Budde, 2009), migration of a Calcofluor method originally implemented in FIA or SFA systems to a 96-well microplate fluorimeter yielded a simple, convenient, and flexible means to analyze the  $\beta$ -glucan content of grains and malt. Figure 1 shows that duplicate standards from 0 to 1.75  $\mu\text{g}$  of  $\beta$ -glucan gives a linear ( $r^2 = 0.99$ ) fluorescence response. Although not fully demonstrated in this data set, the assay response was linear to 3  $\mu\text{g}/200\ \mu\text{L}$  well assay volume and could be further extended by use of a second-order regression fit to data beyond the range of linearity (Schmitt and Budde 2009). Sample analysis shows linear responses to added sample for the sample dilution curve from grain (2–10  $\mu\text{L}$ ,  $r^2 = 0.99$ ) and malt (20–50  $\mu\text{L}$  aliquots,  $r^2 = 0.98$ ) from the experimental barley line BT497 (Fig. 1). One of the useful features of the microplate implementation of the Calcofluor assay is that it readily accommodates different concentrations of  $\beta$ -glucan in barley and malt (Astrup and Jørgensen 1998, table 1) by simply adjusting the aliquot size used in the assay to read within the linear range of the standard curve. To account for the different levels of  $\beta$ -glucan in grain and malt, Astrup and Jørgensen (1988) used a larger (3 $\times$ ) sample size (150 mg vs. 50 mg) during extraction of the lower concentration malt. The flexibility of the microplate  $\beta$ -glucan assay allows somewhat

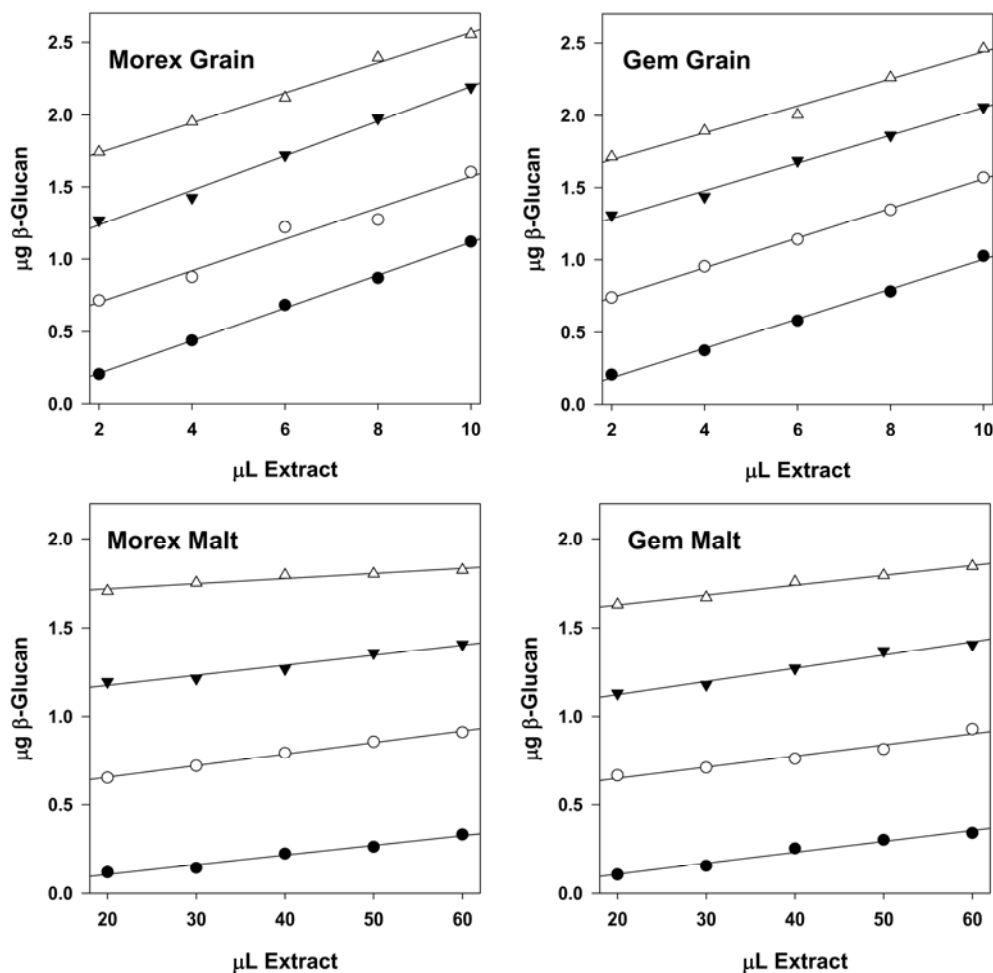
simplified processing through the extraction of consistent sample weights. Similarly, the assay should also accommodate a wide range of sample  $\beta$ -glucan concentrations in atypical samples through adjustment of the aliquot size used, in contrast to the fixed sample injection volume of most FIA/SFA systems.

A second benefit of a microplate version of the Calcofluor assay is that it readily accepts additions of internal  $\beta$ -glucan standards, either for use in calibrating the assay or, as shown by Schmitt and Budde (2009), for identification of interfering compounds in the extract analyzed. Using internal standard additions to wort dilution series, Schmitt and Budde found that there appeared to be constituents in Congress worts that reduced the Calcofluor/ $\beta$ -glucan fluorescent signal, interfering with the assay. Previously, Izawa et al (1996) had also found that unidentified wort components interfered with the Calcofluor/ $\beta$ -glucan signal generation. In contrast, data in Fig. 2 show no indication of similar interfering compounds in acid extracts of barley or oat, grain or malt. The parallel curves seen for the several extract dilution series in the presence of increasing amounts of added barley  $\beta$ -glucan internal standard indicate that no interfering compounds are present under the extraction and assay conditions used. While it is tempting to speculate that the different extraction conditions (aqueous treatment at 100°C for 60 min, followed by acid treatment for 10 min at 100°C used in this study vs. the more gentle temperature ramp not exceeding 70°C for the Congress Mash cycle for wort generation) may have contributed to a differential extraction or stability of the compounds interfering with the wort  $\beta$ -glucan analysis; we have not examined that hypothesis directly in this brief note.

Table I shows the results of  $\beta$ -glucan analyses of two malting barley cultivars (Newdale [2-row] and Morex [6-row]), two oat cultivars (Dane and Gem), along with several experimental malting barley lines determined by FIA and microplate Calcofluor methods. Also included for comparison are the corresponding Congress wort  $\beta$ -glucan values the experimental barley cultivars, taken from Schmitt and Budde (2009, e-Xtra table III). Calculated concentrations of total  $\beta$ -glucan in grain (3–6%) and malt (0.2–1.6%) and soluble  $\beta$ -glucan in wort (10–400 ppm) are comparable to those reported by Astrup and Jørgensen (1988). Pearson correlation coefficients for all pairwise comparisons of the various measurements are shown in Table II. We found very highly significant correlations between results obtained using the FIA and microplate methods on the same sample classes (total  $\beta$ -glucan in grain or malt, or soluble  $\beta$ -glucan in wort). And, as reported by Astrup and Jørgensen (1988), there were generally very good correlations between malt and wort  $\beta$ -glucan values (with all correlations significant at the 1% level).



**Fig. 1.** Microplate-format Calcofluor determination of  $\beta$ -glucan in an extract from barley malt. **A**,  $\beta$ -D-Glucan standard curve ( $r^2 = 0.99$ ). **B**, Dilution series 2–10  $\mu\text{L}$  of acid extract of grain from barley line BT 497 ( $r^2 = 0.99$ ). **C**, Dilution series 20–50  $\mu\text{L}$  of acid extract of malt from experimental barley line BT 497 ( $r^2 = 0.98$ ). Points plotted are duplicate analyses for given concentrations showing repeatability.



**Fig. 2.** Internal standard additions to extracts from Morex barley and Gem oat, for both grain and malt. Symbols: no addition (solid circle), + 0.5 µg of barley β-glucan (open circle), + 1.0 µg of barley β-glucan (solid triangle), and + 1.5 µg barley β-glucan (open triangle).

**TABLE I**  
Grain, Malt, and Wort β-Glucan Concentrations (βG) for Barley (Morex, Newdale, and experimental lines) and Oat (Dane, Gem)  
Determined by Microplate and FIA Calcofluor Methods<sup>a</sup>

Cultivar or Line	Grain %βG (microplate)	Grain %βG (FIA)	Malt %βG (microplate)	Malt %βG (FIA)	Wort ppm βG (microplate) <sup>b</sup>	Wort ppm βG (FIA) <sup>b</sup>
Newdale	4.72	4.31	0.28	0.62	52	80
2ND 19854	4.83	4.66	0.89	0.93	271	330
6B00-1323	4.66	4.23	0.34	0.51	17	46
BT 493	4.87	4.71	0.27	0.55	11	43
ND 20299	5.65	5.30	1.57	1.00	267	383
ND 20303	5.12	5.04	1.16	0.84	181	320
2ND 21863	5.21	4.89	0.63	0.62	77	154
BT 497	5.95	5.29	0.87	0.73	90	143
Morex	3.99	3.79	0.18	0.44	nd	nd
Dane	4.07	4.71	0.78	0.92	nd	nd
Gem	3.71	3.19	0.20	0.59	nd	nd

<sup>a</sup> Values reported for grain and malt are total β-glucan; values reported for wort are water-soluble β-glucan; nd, not determined.

<sup>b</sup> Data from Schmitt and Budde (2008) e-Xtra table 3.

**TABLE II**  
Pearson Correlation Coefficients and Significance Levels (in parentheses) for Microplate and FIA Analysis  
of Barley and Oat Grain, Malt, and Wort Data Shown in Table I

	Grain FIA	Malt Microplate	Malt FIA	Wort Microplate	Wort FIA
Grain microplate	<b>0.8749 (0.0004)</b>	<b>0.6522 (0.0297)</b>	0.3828 (0.2452)	0.3138 (0.4491)	0.3766 (0.3579)
Grain FIA	—	<b>0.7740 (0.0052)</b>	<b>0.6155 (0.0438)</b>	0.5021 (0.2049)	0.5944 (0.1202)
Malt microplate		—	<b>0.8817 (0.0003)</b>	<b>0.8589 (0.0063)</b>	<b>0.9223 (0.0011)</b>
Malt FIA			—	<b>0.9794 (&lt;0.0001)</b>	<b>0.9688 (&lt;0.0001)</b>
Wort microplate				—	<b>0.9746 (&lt;0.0001)</b>

<sup>a</sup> Grain and malt comparisons  $n = 11$ ; wort comparisons  $n = 8$ . Values in bold type are significant at  $\geq 5\%$  level.

One might also expect significant correlations between grain and wort  $\beta$ -glucan measurements, with lines showing high or low  $\beta$ -glucan in the grain tending to produce similar high or low levels of  $\beta$ -glucan in wort after malting and mashing. However, similar trends between grain  $\beta$ -glucan concentrations and wort  $\beta$ -glucan levels are not found, with no significant correlations apparent between grain and wort  $\beta$ -glucan measurements (Table II), as was also reported by Aastrup and Jørgensen (1988). A detailed consideration of the biological processes in germination and malting of grain and subsequent mashing, and how those processes may affect  $\beta$ -glucan pools is beyond the scope of this Note. Aspects of these processes have been variously considered by Bamforth and Martin (1981, 1983), Ellis et al (1997), Rimsten et al (2002), and Wang et al (2004), among others. Because grain development, germination, and enzymatic conversions during mashing all are dependent to some degree on genotypic composition, environmental conditions, and genotype-by-environment interactions, the various processes affecting  $\beta$ -glucan solubilization are sufficiently variable that it is not possible to predict the flux from grain total  $\beta$ -glucan to wort soluble  $\beta$ -glucan. As a result, it is not feasible to predict a final wort  $\beta$ -glucan concentration from grain  $\beta$ -glucan content alone.

### CONCLUSIONS

$\beta$ -Glucan from cereal (barley and oat) grain and malt is easily determined using Calcofluor fluorescence in a microplate fluorimeter. The method is sensitive and scalable to cover a wide range of  $\beta$ -glucan concentrations by simply adjusting the aliquot size used in the 96-well microplates. The microplate assay uses inexpensive reagents and commonly available instrumentation, obviating the need for investment in flow injection analysis instrumentation or commercial reagent kits, providing an attractive alternative to enzymatic kits or flow injection analysis systems.

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